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1. Preliminary phases of a study to determine the probability of release upon impact of viable microorganisms from within solids were completed. A model system was utilized in which methyl methacrylate containing spores of Bacillus subtilis var. niger was polymerized to form a hard, clear, impervious plastic. Quantitative analysis of the spore concentration was achieved by dissolving the plastic in acetone, filtering the solution through a solvent-resistant membrane filter, and plating the filter on trypticase soy agar (TSA). Impact was simulated on a model basis by breaking a plastic disk along a single plane, thereby consistently exposing a measurable internal area of the plastic. The disk fragments were placed in tubes containing trypticase soy broth (TSB) and incubated at 32 C for 30 days. Release of at least one viable microorganism was indicated by the occurrence of visible growth of B. subtilis var. niger in the broth. By fracturing and incubating separately 20 disks from a single batch of plastic, the percent of tubes positive for growth provided a data point approximating the probability of release of at least one viable microorganism from a known surface area of plastic having a known spore concentration.

Experiments to date have provided 280 data points relating probability of release, spore concentration and surface area exposed. The data fit straight-line relationships which could provide a method of estimating the probability of release for any combination of spore concentration and exposed surface area.

2. Efforts to find a satisfactory device for physically measuring the ability of an ultrasonic bath to remove microbial contamination from surfaces continued to be unsuccessful. Accordingly, studies to develop a method of determining the acceptability of a unit for use with the "Standard Procedures for the Microbiological Examination of Space Hardware" were directed toward the utilization of microbiological indicators The most promising approach involves inoculating stainless steel strips with test spores in a standard manner and determining the efficacy of an ultrasonic bath in recovering the test spores when used in a specified standard manner Various combinations of procedures for inoculating stainless steel strips with test spores and subsequently removing them by ultrasonication were studied. Aerosol preparations of an ethanol suspension of spores in a closed chamber followed by deposition of the spores on stainless steel strips appeared to produce an inoculum with the smallest amount of variation between strips (coefficient of variation = 14%) However, at low concentrations some difficulty was experienced in consistently achieving the desired mean values.

A technique was developed by which drops, of approximately 10^{-5} ml, could be placed on stainless steel strips in any number or location desired This was done using a #0 Leroy lettering pen and touching it to a stainless steel surface in a manner similar to making a period. By filling the pen with a water suspension containing 1×10^5 spores per ml each drop would be expected to contain an average of one spore A test in which 31 sets of six strips each were inoculated with 30 drops per strip resulted in a mean deposition of 29 spores per strip. However, the variation was slightly higher than with the aerosol method (coefficient of variation = 23%).

Because ultrasonic baths are manufactured in various configurations, several methods of ultrasonicating the stainless steel strips in a manner applicable to all sizes and shapes of baths were tested. However, additional tests must be conducted before the final selection of a method is made.

3. Comparative studies were continued on the recovery of microorganisms by fresh meat infusion media and trypticase soy agar. It was reported earlier (Report #18) that pork infusion thioglycollate agar (PIT) was approximately three to four times more efficient than TSA for recovering pure cultures of anaerobic spores injured by dry heat This difference was not considered great enough to justify the use of PIT routinely because of the increased amount of time, money, and variation among batches that is associated with its preparation and use. However it was felt that PIT should be field tested in order to determine its efficiency for recovery of naturally occurring microorganisms Two experiments were performed in Phoenix and one at Cape Kennedy The general procedure was to expose 50 to 60 stainless steel strips to the intramural environments of a conference room (Phoenix) and spacecraft assembly area (Cape Kennedy) Each strip was assayed according to the "Standard Procedures for the Microbiological Examination of Space Hardware." However, instead of plating two 5 ml portions, one was plated with TSA and the other with PIT The results (Table 1) showed that in most cases TSA recovered significantly more aerobic and anaerobic microorganisms than PIT. In addition, TSA, in most cases, recovered slightly more or essentially the same number of aerobic and anaerobic spores than PIT Considering the results of these studies and those reported earlier there does not appear to be any justification for replacing TSA with PIT for routine use.

Several experiments were performed to determine the effect of rinse fluid temperature (1% peptone water) on the enumeration of bacterial spores from mixed populations by heat shock. Some investigators feel that some spores may germinate in peptone water if it is held at room temperature for a short time prior to the heat shock. If this were true the spore counts would be reduced markedly In one series of experiments stainless steel strips which had been exposed to a relatively "dirty" area were employed. Each strip was placed in a 4 oz bottle containing 50 ml of 1% peptone water at 4 C and then insonated for 12 minutes in an ultrasonic bath The bottles were placed immediately into an ice bath and the peptone water was divided into two portions One was kept at 4 C and the other at 25 C for 30 minutes prior to being placed in a water bath at 80 C for 20 minutes. Duplicate 5 ml portions were plated with TSA and incubated at 32 C for 72 hours. The results (Table 2) showed that in two experiments the spore counts were two to three times higher when the peptone water was kept at the cold temperature rather than at room temperature In the other two experiments no marked differences in spore counts occurred between peptone water kept cold and at room temperature prior to heat shock. In a similar experiment stainless steel strips, which had been previously exposed to naturally occurring airborne contamination, were processed according to the "Standard Procedures for the Examination of Space Hardware" with the following exception: half of the strips were placed in bottles containing 50 ml of cold (4 C) peptone water, and insonated for 12 minutes in an ultrasonic bath (bath temperature was 18 to 20 C). The suspensions were then heat shocked, and 5 ml portions were plated in quadruplicate with TSA and incubated at 32 C for 72 hours The remaining strips were processed in the standard fashion in which the temperature of the peptone water was 25 C. The suspensions were plated and incubated as described above. There was no marked difference in spore counts between the samples processed at the different temperatures (Table 3).

Since many workers who use the NASA "standard procedures" have suggested that cold peptone water be used in conjunction with spore assays, these studies will be continued at Phoenix and at Cape Kennedy.

4. Studies were continued on the kinetics of dry heat inactivation. Emphasis was placed on determining D_{125C} values for soil samples from various areas in the United States where spacecraft are fabricated, assembled, tested, or launched. It was felt that using naturally occurring spore populations rather than soild isolates which are artificially cultured and sporulated would yield more useful and pertinent information. One-hundred grams of each soil sample were dried for 48 hours at 50 C in a 1-liter Erlenmeyer flask. One-hundred milliliters of 95% ethanol were added to the dried soil, and the suspension was insonated for 30 minutes in an ultrasonic bath to break up clumps of microorganisms and soil. The suspension was filtered through a sterile linen towel to remove excess debris and large soil particles. After this treatment no vegetative bacteria, fungi, or actinomycetes were detected. The remaining microbial flora consisted of bacterial spores. We have shown previously that storage in ethanol does not alter the dry heat resistance of spores.

The tests were performed as follows: sterile stainless steel strips (1/2" x 1/2") were inoculated with 0.05 ml of the soil suspension and dried under vacuum for 16 hours in a desiccator over silica gel. For each of 6 intervals, three strips were suspended in a dry heat oven (forced air) at 125 C for the desired period. Since the strips take 2-1/2 minutes to come up to temperature, this amount of time was added to the exposure interval. For example, if the exposure interval was 25 minutes, the strips were exposed for a total time of 27-1/2 minutes. Immediately after heating the strips were placed in tubes containing 10 ml of chilled, buffered distilled water and several glass beads, and insonated for 12 minutes in an ultrasonic bath. The suspensions were diluted appropriately and plated in triplicate with TSA. One set of strips was used as a control (no heat), and six were heated for the desired times. Three additional sterile strips were processed as sterility controls. Colony counts were made after 48 hours of incubation at 32 C. After several experiments it was observed that diphasic survivor curves were being obtained (Table 4). This phenomenon occurred for all samples. With the exception of the sample from Phoenix, all had a preliminary phase lasting from 10 to 15 minutes and D_{125C} values ranging from 4.7 to 20.3 minutes and secondary phases ranging from 24.5 to 52.1 minutes. The secondary phases had D_{125C} values approximately 2-1/2 to 5 times higher than those of the first phase.

The sample containing the most heat resistant spore flora was the one collected in Phoenix (Sample X). In three experiments the D_{125C} for the first phase was 26.7, 27.9, and 26.7. The D_{125C} for the secondary phases varied depending on the total time of exposure (Table 4, Figure 1). This may have been due to the presence of more than two phases. In any case it is quite evident that there were at lease two spore populations, having separate D_{125C} values.

Colonies surviving from Sample X heated for 120 minutes at 125 C were picked, cultured and sporulated in TAM agar supplemented with $MgSO_4$ and $CaCl_2$. The resultant spore crop was harvested, cleaned and stored in 95% ethanol. Part of this suspension was then mixed with the original soil suspension, which had been centrifuged and dry heat sterilized, so that it contained approximately the same number of spores per unit-volume as did the original soil suspension prior to being sterilized. D_{125C} values were determined for these artificially cultured spores with and without (naked) the presence of soil. The results (Table 5) showed that there was no difference in the D_{125C} values and that the survivor curves were monophasic. Consequently the soil _per se_ did not seem to alter heat resistance by physical protection. In addition the D_{125C} values of the artificially cultured spores were much less than those of the second phase of the original soil suspension from which they were isolated. This indicates that the naturally occurring spores lost some heat resistance after being grown on artificial culture media.

The diphasic survivor curves did not appear to be the results of technique-induced artifacts. All pure cultures of spores including _B. subtilis_ var. _niger_, _B. cereus_ T. _B subtilis_ 5230 and all soil isolates have produced monophasic curves. In addition the soild suspensions did not appear to alter the heat resistance or rate of destruction of the spores with which it was associated.

These studies will be continued and samples from other areas will be examined.

5. Studies on levels of microbial contamination in the intramural environments of the Hangar AO clean room, area 60-A sterilization and assembly laboratory, and the Surveyor fuel loading room were continued. Similar studies were initiated in the various Apollo facilities. Figure 2 depicts the Apollo areas, including the spacesuit and life support system, and the movement of spacecraft through them. Levels of airborne contamination in Apollo areas are presented in Tables 6, 7 and 8. Table 9 shows the levels of microorganisms accumulating on stainless steel strips exposed in the integrated test stand #2, the spacesuit clean room, and the backpack life support system clean room.

   Swab samples were taken from Lunar Orbiter 3, Surveyor 4, A-IMP-E, Surveyor 5 and Surveyor 6. Results are shown in Tables 10, 11 and 12.

6. Work has resumed on the bioclean room and acceptance testing began the last week in September. The filter framing system for the ceiling has been completely redesigned. The original framing system and filters have been removed and will be replaced in accordance with the new design.

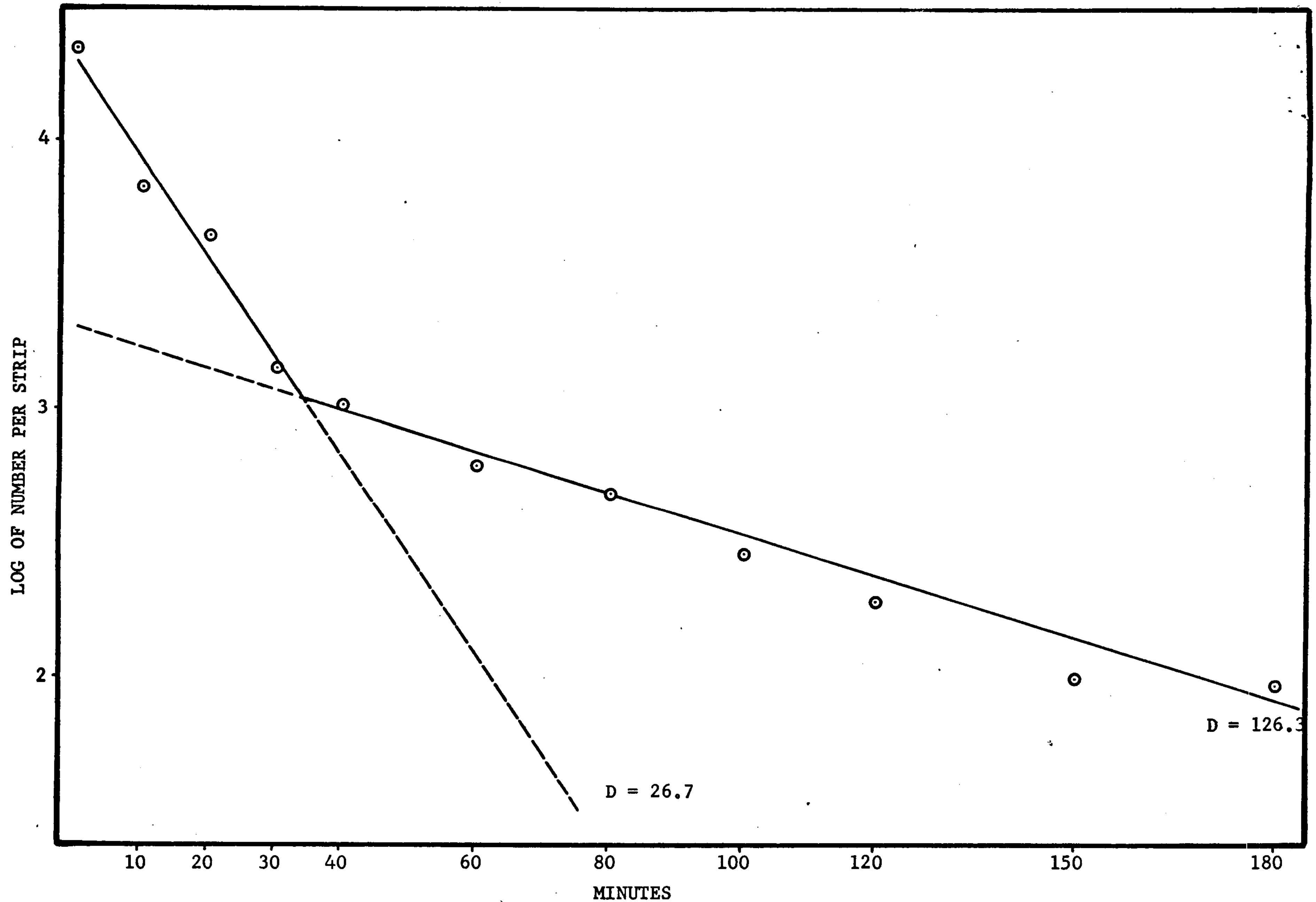

FIGURE 1. DEATH RATE OF NATURALLY OCCURRING SPORE FLORA IN SOIL SAMPLE X AT 125 C.

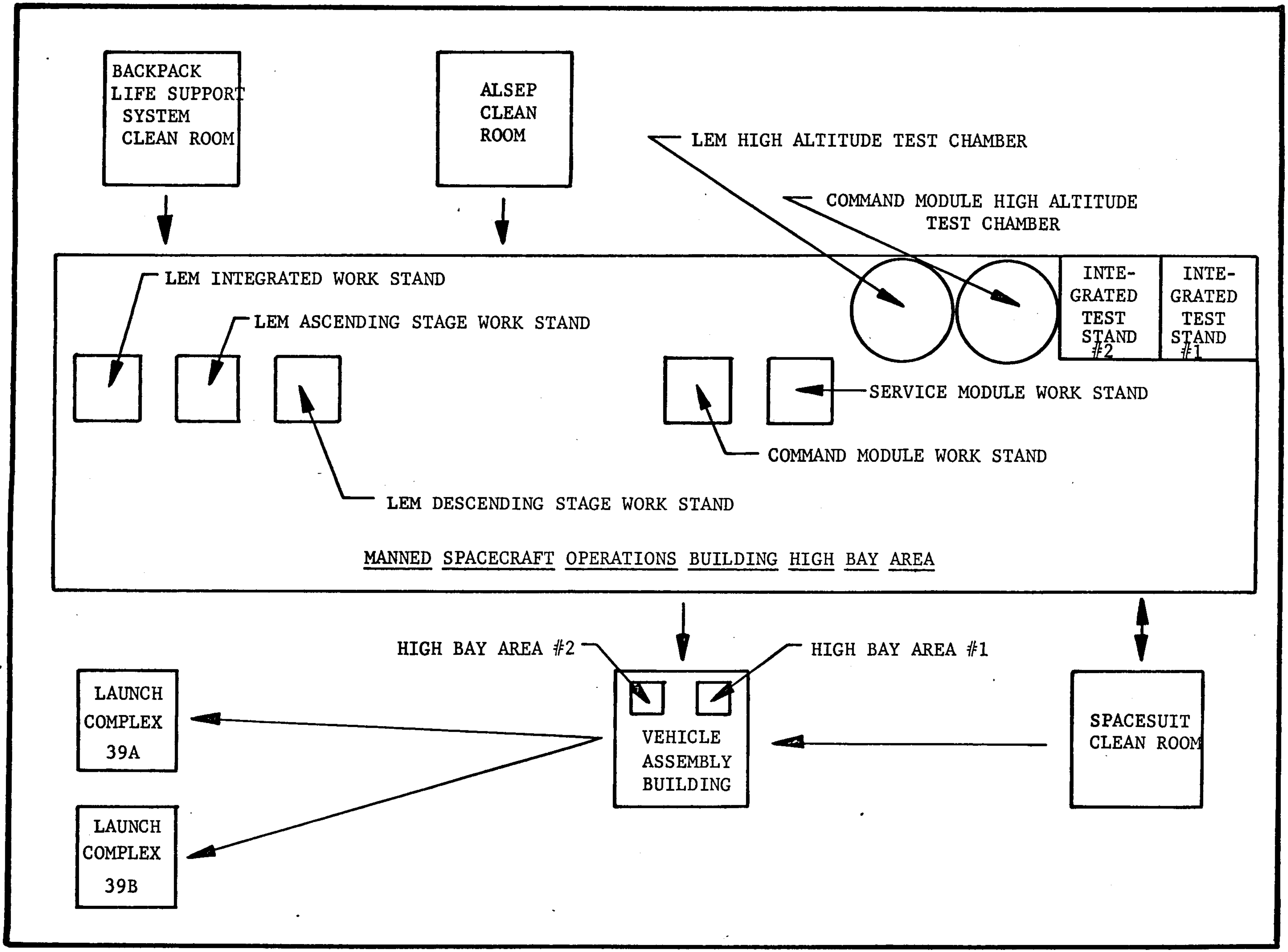

FIGURE 2. FACILITIES ASSOCIATED WITH THE APOLLO PROGRAM.

TABLE 1. COMPARATIVE RECOVERY OF NATURALLY OCCURRING AIRBORNE MICROORGANISMS ON STAINLESS STEEL STRIPS WITH TRYPTICASE SOY AGAR AND PORK INFUSION THIOGLYCOLLATE AGAR.

Type of microorganisms	Medium[1]	No. of tests	No. of microorganisms per strip: Mean	Range	P[2]
			EXPERIMENT 1, PHOENIX		
Aerobes	TSA	13	47.8	16-228	< 0.05
	PIT		22.8	11-77	
Aerobic spores	TSA	20	6.6	1-14	NSD[3]
	PIT		6.1	2-12	
Anaerobes	TSA	49	9.9	0-51	< 0.001
	PIT		5.6	0-19	
Anaerobic spores	TSA	46	2.5	0-20	NSD
	PIT		2.1	0-5	
			EXPERIMENT 2, PHOENIX		
Aerobes	TSA	33	55.3	10-266	< 0.001
	PIT		26.7	6-108	
Aerobic spores	TSA	43	2.3	0-11	NSD
	PIT		2.4	0-11	
Anaerobes	TSA	52	14.6	0-421	< 0.01
	PIT		11.6	0-424	
Anaerobic spores	TSA	52	0.77	0-5	NSD
	PIT		0.86	0-5	

TABLE 1 (CONT'D.) COMPARATIVE RECOVERY OF NATURALLY OCCURRING AIRBORNE MICROORGANISMS ON STAINLESS STEEL STRIPS WITH TRYPTICASE SOY AGAR AND PORK INFUSION THIOGLYCOLLATE AGAR.

Type of microorganisms	Medium[1]	No. of tests	No. of microorganisms per strip Mean	Range	P[2]
			EXPERIMENT 3, CAPE KENNEDY		
Aerobes	TSA	44	92.3	5-660	< 0.001
	PIT		58.7	4-316	
Aerobic spores	TSA	51	3.3	0-10	< 0.001
	PIT		5.4	0-16	
Anaerobes	TSA	62	19.4	0-178	< 0.02
	PIT		11.0	0-99	
Anaerobic spores	TSA	62	1.4	0-19	NSD
	PIT		1.5	0-22	

1 TSA - trypticase soy agar; PIT - pork infusion thioglycollate agar.

2 $P < 0.05$ based on student *t* test is considered significant.

3 NSD - no significant difference.

TABLE 2. EFFECT OF RINSE WATER TEMPERATURE DURING STORAGE AND PRIOR TO HEAT SHOCK ON THE RECOVERY OF BACTERIAL SPORES.

Experiment	No. of tests	Mean colony count	
		Cold[1]	Room temperature[2]
1	10	30.0	9.9
2	10	23.6	9.5
3	11	39.7	40.3
4	6	43.1	37.0

[1] Each strip was placed in 50 ml of 1% peptone water which was kept cold during ultrasonication (12 minutes) and for 30 minutes prior to being placed in a water bath at 80 C for 20 minutes.

[2] The same as above except that the temperature of the peptone water was maintained at 25 C for 30 minutes after ultrasonication and prior to heat shock.

TABLE 3. COMPARATIVE SPORE COUNTS OF NATURALLY OCCURRING AIRBORNE MICROBIAL POPULATIONS ON STAINLESS STEEL STRIPS WHEN COLD (4 C) AND WARM (25 C) PEPTONE WATER WERE USED AS THE RINSE FLUID.

Rinse fluid	Mean colony count[1]	Range
Cold	3.7	0.5 - 25.5
Warm	2.3	1.0 - 6.2

[1] Twenty-four strips were processed at each temperature. The rinse fluid from each strip was heat shocked for 15 minutes (plus 5 minutes for come-up time) and plated in quadruplicate with trypticase soy agar.

TABLE 4. COMPARATIVE D_{125C} VALUES FOR NATURALLY OCCURRING BACTERIAL SPORES FROM SOIL SAMPLES COLLECTED FROM VARIOUS AREAS OF THE UNITED STATES[1].

Sample	Source	1st Phase D_{125C}	1st Phase Duration (minutes)	2nd Phase D_{125C}	2nd Phase Duration (minutes)
A	Cape Kennedy, Fla.	4.7	0-10	25.9	10-30
D	The Boeing Co., Seattle	5.8	0-10	24.5	10-30
G	McDonnell Aircraft Corp. St. Louis, Mo.	11.4	0-15	37.6	15-30
H		10.7	0-15	28.2	15-30
I		20.3	0-15	52.1	15-30
J	Langley Research Center Hampton, Va.	10.0	0-15	36.0	15-30
K		7.6	0-15	--[2]	--
L	Marshall Space Flight Center Huntsville, Ala.	10.0	0-10	27.0	10-30
M		11.4	0-10	42.0	10-30
X	Phoenix, Arizona	26.7	0-30	62.5	30-60
X		27.9	0-40	140	40-120
X		26.7	0-30	126.3	30-240

1 For all experiments stainless steel surfaces (1/2 x 1/2") were suspended within a dry heat oven at 125 C.

2 The number of survivors was too low for valid colony counts.

TABLE 5. D_{125C} VALUES OF SPORES ISOLATED FROM HEATED[1] SOIL SAMPLE X, CULTURED AND SPORULATED ON ARTIFICIAL CULTURE MEDIA AND HEATED SEPARATELY OR IN THE PRESENCE OF STERILE SOIL.

Experiment	Inoculum	D_{125C} Value	
		Mean	95% confidence limits
1	In soil[2]	46.9	39.2 - 58.5
	Naked[3]	49.8	41.5 - 62.1
2	In soil	37.7	31.3 - 47.4
	Naked	40.2	31.8 - 54.3

[1] Isolates were taken from colonies which developed from survivors of soil sample X after being exposed to dry heat at 125 C for 120 minutes.

[2] An ethanol suspension of spores, originally cultured and sporulated on artificial culture media (TAM), was added to soil suspension X which had been sterilized by dry heat. The spore-soil suspension was stored for 3 days at 4 C prior to inoculation.

[3] The same spores as described above were used except that they were not mixed with soil.

TABLE 6. AIRBORNE VIABLE PARTICLES PER FIVE CUBIC FEET WITHIN THE MANNED SPACECRAFT OPERATIONS BUILDING HIGH BAY AREA.

	Sites[1]									
Time	1	2	3	4	5	6	7	8	9	10
0930	13.1	16.3	4.7	6.9	3.3	6.3	4.8	2.1	12.8	7.9
1030	15.3	17.5	5.4	6.3	3.6	8.9	3.5	3.8	14.6	8.1
1130	11.3	11.6	5.9	4.1	4.8	6.0	3.8	3.2	14.3	17.4
1230	17.9	19.1	4.3	3.8	3.0	7.2	3.5	3.1	10.9	7.0
1330	--	--	4.3	5.3	2.8	9.6	4.2	3.2	14.5	16.3
1430	--	--	4.8	4.5	3.8	13.0	5.3	5.3	11.2	9.0
AVERAGE	14.4	16.1	4.9	5.15	3.55	8.5	4.18	3.45	13.1	10.5

[1] Distance between sites was 50 feet.

TABLE 7. AIRBORNE VIABLE PARTICLES PER FIVE CUBIC FEET WITHIN VARIOUS APOLLO AREAS.

	Location			
Time	LEM[1] ascent stand[2]	LEM descent stand[2]	Spacesuit clean room	Back pack clean room
0930	6.4	14.0	1.6	7.1
1030	7.9	16.5	8.5	2.9
1130	11.2	23.4	16.0	1.7
1230	9.9	11.5	1.4	0.4
1330	10.3	15.7	16.6	1.4
1430	10.8	12.5	1.7	2.3
AVERAGE	9.4	15.6	7.6	2.6

[1] LEM - Lunar Excursion Module

[2] Air samplers were 16 feet above floor level.

TABLE 8. AIRBORNE VIABLE PARTICLES PER FIVE CUBIC FEET WITHIN FOUR APOLLO AREAS.

	Location							
	LEM[1] high altitude test chamber		Command module high altitude test chamber		Service module work stand		Command module work stand	
Time	Site 1	Site 2[2]	Site 1	Site 2[3]	Site 1	Site 2[3]	Site 1	Site 2[2]
0930	4.5	3.8	14.2	15.6	3.8	3.8	4.3	7.7
1030	8.3	5.7	16.8	26.5	2.9	---	4.6	7.2
1130	12.9	49.7	20.2	22.5	3.8	3.3	4.5	8.8
1230	11.0	24.8	11.5	8.3	10.4	4.9	3.7	6.2
1330	19.9	24.7	14.5	14.0	18.7	7.2	6.2	8.2
1430	12.0	43.1	15.3	17.9	14.1	8.1	5.5	8.3
AVERAGE	11.4	25.3	15.4	17.5	9.0	5.5	4.8	7.7

1 LEM - Lunar Excursion Module

2 Air samplers were 16 feet above floor level.

3 Air samplers were 20 feet above floor level.

TABLE 9. MICROBIAL CONTAMINATION ACCUMULATING ON STAINLESS STEEL STRIPS EXPOSED WITHIN VARIOUS APOLLO AREAS.

Location	Exposure	Aerobes no./ft.^{2}	Anaerobes no./ft.^{2}	Aerobic spores no./ft.^{2}	Anaerobic spores no./ft.^{2}
MSOB[1] integrated test stand #2	1 week				
	average	4,980	480	0	0
	range	0-10,800	0-1,440	--	--
	3 weeks				
	average	372,240	23,130	93,840	24,336
	range	29,520-543,600	4,680-50,400	3,600-114,120	360-101,520
Spacesuit clean room	1 week				
	average	780	60	60	0
	range	0-1,800	0-360	0-360	--
	2 weeks				
	average	960	240	60	60
	range	360-2,520	0-360	0-360	0-360
	3 weeks				
	average	1,858	60	0	60
	range	720-4,680	0-360	--	0-360
Back pack clean room	1 week				
	average	360	60	0	0
	range	0-1,440	0-360	--	--
	2 weeks				
	average	1,980	240	180	0
	range	360-3,960	0-720	0-360	--
	3 weeks				
	average	2,095	0	0	0
	range	0-7,200	--	--	--

[1] MSOB - Manned Spacecraft Operations Building

TABLE 10. MICROBIAL CONTAMINATION DETECTED ON THE SURFACES OF LUNAR ORBITER 3, LUNAR ORBITER 3 SHROUD, SURVEYOR 4 AND SURVEYOR 4 ADAPTER

Source	Date	Area sampled[1] (sq. in.)	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Lunar Orbiter 3						
spacecraft	7-3-67	57.1	160	70	30	20
shroud	7-5-67	80	15	5	40	0
spacecraft	7-11-67	57.1	155	55	5	0
spacecraft	7-21-67	57.1	595	5	30	0
Surveyor 4						
(prior to cleaning)						
spacecraft	7-7-67	80	3,440	850	0	0
adapter	7-7-67	80	5,735	1,435	35	0
(after cleaning)						
spacecraft	7-8-67	80	130	30	0	0
adapter	7-8-67	80	1,680	95	0	5

[1] Swab-rinse technique

TABLE 11. MICROBIAL CONTAMINATION DETECTED ON THE SURFACE OF A-IMP-E SPACECRAFT

Source	Date	Area sampled[1] (sq. in.)	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
A-IMP-E[2]						
solar paddle	7-12-67	91.2	10	0	10	0
solar paddle	7-13-67	136.8	30	5	5	5
(prior to cleaning)						
A-IMP-E spacecraft	7-17-67	244	1,250	225	170	5
(after cleaning)						
spacecraft	7-17-67	244	495	---	0	10

1 Swab-rinse technique

2 A-IMP-E - Anchored Interplanetary Monitoring Platform E

TABLE 12. MICROBIAL CONTAMINATION DETECTED ON THE SURFACES OF THE SURVEYOR 5 SPACECRAFT, ADAPTER, AND SURVEYOR 6 SPACECRAFT

Source	Date	Area sampled[1] (sq. in.)	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Surveyor 5 spacecraft	6-29-67	80	25	30	35	15
	7-24-67	80	100	60	10	15
	8-11-67	80	1,015	310	5	0
Surveyor 5						
retro rocket	8-23-67	80	1,800	745	5	0
AMR[2]	8-23-67	80	930	805	70	10
(prior to cleaning)						
spacecraft	8-31-67	80	8,085	1,865	50	0
adapter	8-31-67	40	1,810	540	5	0
(after cleaning)						
spacecraft	8-31-67	80	220	130	0	0
adapter	8-31-67	72	2,500	360	0	0
Surveyor 6 spacecraft	9-12-67	80	10	10	30	15

[1] Swab-rinse technique

[2] AMR - Altitude Marking Radar